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(54) Title: RECOMBINANT ADENOVIRAL VECTOR EXPRESSING ANTIGENS ASSOCIATED WITH COLORECTAL TUMORS

(57) Abstract

The present invention describes a way of presenting colorectal tumor-associated antigens to the immune system of a host by employing recombinant adenovirus. The recombinant adenoviruses provided by the present invention are useful for tumor treatment and prevention.

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# RECOMBINANT ADENOVIRAL VECTOR EXPRESSING ANTIGENS ASSOCIATED WITH COLORECTAL TUMORS

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## TECHNICAL FIELD OF THE INVENTION

The present invention is related to a recombinant adenovirus expressing an antigen associated with colorectal tumors. Such recombinant adenoviruses are useful in inhibiting tumor growth as well as inducing protective immunity against tumor cells, even those without the antigen encoded in the recombinant adenoviruses.

## BACKGROUND OF THE INVENTION

Currently available treatments for cancer, particularly radiation therapy and chemotherapy, are based upon the rationale that cancer cells are relatively more sensitive to these treatments than normal cells. However, severe toxicity for normal tissues imposes major limitations on these therapies. In contrast, antibody molecules exhibit exquisite specificity for their antigens. Researchers have therefore sought to isolate antibodies specific to cancer cells for cancer therapy.

Passive immunotherapy of cancer patients with antibodies against tumor antigens has been tested in clinical trials and has shown promising results. For example, the

monoclonal antibody against the antigen associated with gastrointestinal cancers, mAb CO17-1A, has been administered to cancer patients in total doses of up to 10 g per patient and has been well tolerated, despite the expression of the antigen on some normal tissues (1-4). A recent phase II trial with 189 patients randomized to treatment with mAB CO17-1A or placebo control and a median follow-up of 5 years has demonstrated a significant ($p<0.01$) increase in survival of mAB-treated versus control patients (5).

Active specific immunotherapy is designed to enhance immunologic response of patients to their own tumors. Attempts to develop active immunotherapies for patients with cancer have involved extracts from cancer cells, intact tumor cells, or gene-modified tumor cells administered either alone or with immune adjuvants (6). The recent identification of the genes encoding tumor-associated antigens such as MAGE-1 (7), MAGE-3 (8), tyrosinase (9), MART1/Melan-A (10, 11, 12), gp100 (13), and others has opened new possibilities for the development of cancer treatment and cancer prevention.

Tumor-associated antigens can be introduced into a host's immune system via different routes. One way to present tumor-associated antigens to the host's immune system is to employ anti-idiotypic antibodies (Ab2) that bind to the antigen-combining site of an antitumor antibody (Ab1) (14, 15). These anti-idiotypic antibodies (Ab2) may functionally mimic the tumor-associated antigen defined by the antitumor antibody (Ab1). The advantages of Ab2s are their high specificity, ease of production, and potential to break the immunologic tolerance to tumor-associated antigens commonly found in cancer patients. However, an Ab2 may only mimic one epitope on a tumor-associated antigen. A tumor-associated antigen expresses multiple potentially immunogenic epitopes and may

thereby elicit more effective anti-tumor immunity than an Ab2.

Another way to present tumor-associated antigens is to use recombinant viral vectors expressing the antigens. In mice, active tumor immunotherapy using EBV envelope gp340/220 (16), human melanoma-associated antigen gp100 (17), and the model tumor antigen β-galactosidase (18) as the replication-defective recombinant adenovirus-encoded antigen has been described. EBV-associated protein and β-galactosidase are xenogeneic antigens in mice, and an antigen homologous to human gp 100 has yet to be characterized in detail (19). Since most tumor antigens are also expressed on normal tissues (20, 21), such xenogeneic model antigens may not provide reliable information of the immune response.

Although in the xenogeneic β-galactosidase model antigen system (16), the adenovirus (Ad2) expressing the antigen inhibited growth of 3-day-old established pulmonary experimental metastases, IL-2 was required for significant anti-tumor effects, and the observation period was only 2 weeks. Furthermore, the vaccine was injected i.v. into animals with pulmonary metastases, raising the question of whether the vaccine would have been protective when given at a site separate from the tumor, as is usually the case in cancer patients.

Ad EBV-gp340/220 (16) and Ad gp100 (17) protected mice against subsequent challenge with the respective antigen-positive tumor. However, induction of immunity to the tumors without the specific antigen encoded by the recombinant adenovirus has not been reported.

Since most tumor-associated antigens are expressed by less than 100% of the cells

within a tumor lesion, induction of a secondary vaccine effect, *i.e.*, an immunity to the cells of the syngeneic tumors which do not display the tumor-associated antigen initially used as vaccine, is desirable. Such secondary vaccine effect or "antigen spreading" can prevent the outgrowth of antigen-negative escape variant tumor cells. Therefore, there is still a need in the art to develop a new method for introducing tumor-associated antigens into a host system and for inducing more protective immunity against tumors.

## SUMMARY OF THE INVENTION

It is an object of the invention to provide a composition useful for the treatment or prevention of colorectal tumors in a human.

It is another object of the invention to provide a method of inhibiting growth of a colorectal tumor.

It is yet another object of the invention to provide a method of inducing protective immunity against a colorectal tumor cell in a human.

These and other objects of the invention are provided by one or more of the embodiments described below.

In one embodiment of the invention a composition useful for the treatment or prevention of colorectal tumors in a human is provided which comprises a recombinant adenovirus which contains a DNA fragment operably linked to a viral promoter wherein the DNA fragment encodes an antigen associated with a colorectal tumor.

In another embodiment of the invention a method of inhibiting growth of a colorectal tumor is provided which comprises administering to a human in need of such treatment an immunological response-inducing amount of the recombinant adenovirus of

the present invention, wherein the colorectal tumor expresses the antigen encoded by said recombinant adenovirus.

In yet another embodiment of the invention a method of inducing protective immunity against a colorectal tumor cell in a human is provided which comprises administering to a human in need of such treatment an immunity-inducing amount of the recombinant adenovirus of the present invention.

These and other embodiments of the invention provide the art with a new way of presenting tumor-associated antigen to the immune system in a host. The tumor-associated antigen encoded by a recombinant adenovirus has an increased antigen load to the immune system as compared to an isolated antigen protein. Such antigen can also express multiple potentially immunogenic epitopes. The recombinant adenoviruses provided by the present invention inhibit growth of colorectal tumors as well as induce long lasting protective immunity against colorectal tumors. In particular, the immune response that is directed to the antigen-positive colorectal tumors is followed by induction of immunity to tumors that do not express the antigen encoded in the recombinant adenovirus. Therefore, the recombinant adenoviruses of the present invention are useful therapeutic agents to treat tumors and to prevent tumor occurrence as well as reoccurrence.

## BRIEF DESCRIPTION OF THE DRAWINGS

**FIGURE 1.** Binding of anti-Ad5 GA733-2 antibodies to GA733-2 antigen-positive CRC cells. BALB/c mice (4-5 per group) were immunized i.p. with $0.75 \times 10^8$ pfu of Ad5 GA733-2 (●) or control virus Ad5 ΔE3 (■). Sera were collected before immunization and

on various days thereafter. Binding of sera (1:200 dilution) to GA733-2 antigen-positive human CRC cells SW1116 (—) and mouse CRC cells CT26-GA733-2 (– –) was tested in MHA. Post-immunization sera did not bind to GA733 antigen-negative human melanoma cells WM164 or mouse CRC cells CT26. Mean ± S.E. (bars) of 4-5 mice per group. Asterick (*), values differ significantly ($p < 0.05$) from the values in the corresponding control group.

**FIGURE 2.** Binding of anti-Ad5 GA733-2 antibodies to GA733-2E antigen. BALB/c mice (5 per group) were immunized i.p. once with $0.75 \times 10^8$ pfu of Ad GA733-2 (●) or control Ad5 ΔE3 (■). Pre-immune (– –) or post-immune (—) sera were tested for binding to baculovirus-produced GA733-2E antigen in ELISA, using peroxidase-labeled goat-anti-mouse F(ab')$_2$ antibodies to detect binding of murine antibodies to the target. Sera (1:50 dilution) did not bind to BSA (results not shown). Mean ± S.E. (bars) of 5 mice per group. Asterick (*), values differ significantly ($p < 0.05$) from the corresponding values obtained with each of the 3 control groups.

**FIGURE 3.** Antibody binding inhibition. Human SW1116 CRC cells were incubated for 90 min at room temperature with sera (diluted 1:50) from mice (5 per group) immunized once with $0.75 \times 10^8$ pfu of Ad5 GA733-2 or Ad5 ΔE3. Following incubation, $^{125}$I-mAb CO17-1A, GA733, or M77 directed to the GA733 antigen, or CO29-10 control mAb directed to a different CRC-associated antigen, were added. Inhibition of $^{125}$I-mAb binding to cells was calculated relative to buffer controls. Binding of each of the $^{125}$I-labeled mAb to tumor cells was inhibited >85% by ~20 μg/ml of the homologous unlabeled mAb (not shown). Mean ± S.E. (bars) of 5 mice per group. Asterick (*), value

is significantly different ($p < 0.05$) from the corresponding control value.

**FIGURE 4.** Lymphocyte proliferation responses in Ad5 GA733-2 immunized mice. Splenocytes from mice immunized i.p. three times at 1-month intervals with 5 x $10^7$ pfu of Ad5 GA733-2 (●) or control Ad5 ΔE3 (■) were stimulated 60 days after the last immunization with different concentrations of GA733-2E antigen derived from baculovirus (—) or with BSA (– –). [³H]TdR incorporation by the splenocytes was determined after 3 days of incubation, and stimulation indices (SI) were calculated. Mean ± S.E. (bar) of 3 mice per group. Asterick (*), values are significantly different ($p < 0.05$) from the corresponding control values.

**FIGURE 5.** CTL responses in Ad5 GA733-2 immunized mice. Splenocytes from mice immunized i.p. three times at 1-month intervals with 5 x $10^7$ pfu of Ad5 GA733-2 (●) or control Ad5 ΔE3 (■) were stimulated 60 days after the last immunization with autologous splenocytes infected with Ad5 GA733-2 or control Ad5 ΔE3, respectively. The specific lytic activities of the splenocytes against murine CRC cells CT26-GA733-2 (—) and CT26 (– –) were measured in a 4 h $^{51}$Cr-release assay after 5 days in culture. Mean ± S.E. (bar) of 3 mice per group. Asterick (*), values are significantly different ($p < 0.05$) from the corresponding values obtained with either of the 3 control groups.

**FIGURE 6.** Prevention of tumor growth by Ad5 GA733-2. Mice (10/group) were immunized with 1.2 x $10^8$ pfu of Ad5 GA733-2 (●) or control Ad5 ΔE3 (■) twice at 3-week intervals. Five days after the last immunization, mice were challenged with 5x $10^7$ murine CRC cells CT26-GA733-2. Tumor sizes ($mm^2$) were measured bi-weekly. Mean ± S.E. (bar) of 10 mice per group. Asterick (*), values are significantly different ($p < 0.001$

- < 0.01) from the corresponding values obtained from the control group. Ratios indicate number of mice with tumors to total number of mice.

**FIGURE 7.** Prevention of tumor growth and survival enhancement by simultaneous administration of Ad5 GA733-2. Mice were injected on day 0 with 20x $10^6$ murine CRC cells CT26-GA733-2. On days 0 and 7, mice were immunized with 1.2 x $10^8$ pfu of Ad5 GA733-2 (●) or control Ad5 ΔE3 (■). (*A*) Tumor sizes ($mm^2$) were measured bi-weekly. Mean ± S.E. of 10 mice per group. Asterick (*), values are significantly ($p < 0.001$ - < 0.01) different from the corresponding control values. Ratios indicate number of mice with tumors to total number. (*B*) Survival rates of experimental (—) and control (– –) mice. Differences in survival are significant ($p < 0.05$).

**FIGURE 8.** Immunization with Ad5 GA733-2 after challenge with CT26-GA733-2 induces long-lasting protective immunity to CT26 cells. (*A*) Survival enhancement of mice with established CT26-GA733-2 tumors by Ad5 GA733-2. Mice (10 per group) were injected with 20 x $10^5$ murine CRC cells CT26-GA733-2 on day 0. On days 5 and 12, mice were immunized with 1.2 x $10^8$ pfu of Ad5 GA733-2 (—) or control Ad5 ΔE3 (– –). Differences in survival between experimental and control mice are significant ($p < 0.05$). (B) Crossreactive protective immunity against CT26 cells. Five mice whose CT26-GA733-2 tumors had regressed after immunization with Ad5 GA733-2 on days 5 and 12 after tumor inoculation (see *A*) were rechallenged with $10^5$ CT26 cells on day 98 (—). Five naive mice (– –) were injected with $10^5$ CT26 cells. Differences in survival between experimental (—) and control (—) mice are significant ($p < 0.01$).

## DETAILED DESCRIPTION OF THE INVENTION

The present invention is directed to a recombinant adenovirus expressing an antigen associated with colorectal tumors. Such recombinant adenoviruses are particularly useful for treatment and prevention of colorectal tumors.

Generally, colorectal tumors express one or more antigens at a significantly higher level than a normal tissue. According to the present invention, a DNA fragment encoding an antigen associated with a colorectal tumor may be introduced into a patient through a recombinant adenovirus. Such recombinant adenoviruses infect host cells so that the foreign antigen is endogenously produced by the cells and presented to the host immune lymphocytes. The recombinant adenoviruses of the present invention induce humoral and cellular immunity in the host immune system and can be used to inhibit the growth of a colorectal tumor expressing the particular antigen encoded by the recombinant adenovirus. The recombinant viruses described in the present invention may also be used to induce protective immunity against a colorectal tumor and be employed to prevent tumor occurrence or reoccurrence. Preferably a colorectal tumor to be protected against by the recombinant adenoviruses of the present invention carries the same antigen encoded by the recombinant adenovirus. However, protective immunity may also be rendered to colorectal tumors that do not express the specific antigen encoded by the recombinant adenovirus subsequent to a growth reduction or destruction of a tumor carrying the recombinant adenovirus-encoded antigen,. Though not wishing to be limited to any particular mechanism of action, it is postulated that when an immune response causes tumor cell death, the tumor cells may release many tumor antigens other than the specific antigen that

triggers the initial immune response. The tumor antigens may be presented to the immune system through tumor debris and induce a subsequent immune response, *i.e.*, secondary vaccine effect or "antigen spreading". Such response provides a protective immunity against not only the specific tumor antigen which induces the initial response, but also the other antigens released upon tumor cell death.

A broad range of colorectal tumors or tumors containing colorectal tumor antigen(s) can be treated and prevented according to the present invention. Colorectal tumors include Familial adenomatous polyposis, Gardner's syndrome, colorectal adenocarcinoma, and any neoplasms of the large intestine. Tumors carrying colorectal tumor-antigens include, but are not limited to, gastric and pancreatic carcinomas. The tumors may be primary, metastic, actively growing, or stabilized.

Any antigen associated with colorectal tumors is contemplated in the present invention. Colorectal tumor-associated antigens are targets of antibodies and/or T cells in the anti-tumor immune response. They usually are expressed preferentially with a high amount on colorectal tumor cells and not in significant amounts in normal tissues. Generally, colorectal tumor-associated antigens are expressed at about anywhere from 1.5:1 to 5:1 ratio in tumor cells as compared to normal cells. Techniques for detecting antigen expression are well known in the art, *e.g.*, Northern blotting, Western blotting, immunoprecipitation, flow cytometry, *in vivo* immunoscintigraphy (imaging) etc. The *in vivo* immunoscintigraphy (imaging) technique is especially informative of antigen expression. Generally, the full-length of a colorectal tumor antigen is employed for the present invention. Alternatively, epitopes preferentially expressed in colorectal tumor cells

can be employed.

Usually, antigens associated with colorectal tumors are identified through antibodies isolated or derived from the serum, B cells, or T cells of a patient with a colorectal tumor. Techniques for isolating tumor-associated antigens are known and readily available in the art. For example, the antibody phage display method can be used to identify tumor-associated cell surface antigens. A tumor patient's antibody library can be expressed on the surface of filamentous phages. Such antibody-phage library can be screened once or several times by first absorbing antibody-phages with normal cells and subsequently binding by tumor cells. An antibody that specifically binds to tumor cells can be employed to form immuncomplexes with the antigens in the tumor cell extracts. Normally the antibody is linked with a conjugate such as biotin so that the isolation of antibody-antigen complex can be achieved via a compound such as Streptavidin-agarose beads binding to the antibody conjugate. The antigen may then be purified and sequenced by routine experimentation. Many colorectal tumor-associated antigens have been isolated and are readily available in the art, *e.g.*, CO17-1A/GA733 antigen (Szala et al., PNAS 87, pp3542-6, 1990) and CEA (Tsang et al., Journal of the National Cancer Institute, Vol. 87, pp982-990, 1995).

Adenoviruses including all different strains can be used for the purpose of the present invention. Preferably, strains 2 and 5 can be used in the present invention. Such viruses have been employed intensively as vectors for various purposes, *e.g.*, gene transfer. Adenoviruses and vectors containing adenovirus genome are well known and readily available in the art. A recombinant adenovirus of the present invention can be constructed by integrating a DNA fragment encoding a colorectal tumor-associated antigen into the

virus genome. Such a DNA fragment can be inserted at an adenoviral region that is disposable for adenovirus or is not desirable for the purpose of the present invention. Examples of such a region is the adenoviral early region 3 (E3), early region 1 (E1), and early region 4 (E4). E3 down regulates the surface expression of class I MHC antigens and therefore may hamper the antigen presentation of the infected cell. The DNA fragment may be operably or covalently linked in a *cis* configuration to a viral promoter so that expression of the antigen is driven by the viral promoter. The expression of the DNA fragment may be directed by any viral promoter, *e.g.*, early viral promoter or late viral promoter. Preferably the viral promoter is an adenoviral promoter. Viral promoters, especially adenoviral promoters, are known and readily available in the art, *e.g.*, E1A, E1B, E4, and E3. The promoter may be linked with one or more copies of DNA encoding the same or different antigens. Alternatively, several copies of same or different antigens linked with different promoters may be used. These promoter and antigen combinations may be used to increase the expression and presentation of the antigen. A recombinant adenovirus of the present invention may be replication defective or replication competent.

Normally, a recombinant transfer vector is constructed for making a recombinant adenovirus. For example, a DNA fragment encoding an antigen associated with colorectal tumors may be covalently linked to a viral promoter such as the E3, E1A, E1B, and E4 promoter, via methods known in the art, *e.g.*, restriction digestion, polymerase chain reaction (PCR), and ligation. Subsequently, the DNA fragment including the promoter and the antigen may be inserted at the site of a viral gene such as E3, which is carried by a readily available adenoviral plasmid vector, *e.g.*, pAd5. In order to integrate the DNA

fragment into the virus, the recombinant transfer vector may be co-transfected with an adenovirus into a human or animal cell line via methods used in the art, *e.g.*, the lipofection method. Recombinant adenoviruses generated by recombination between the transfer vector and the adenovirus may be determined via detecting the inserted antigen. The detection may be by Southern blot, Northern blot, PCR, Western blot, restriction digestion, FACS analysis of virus-infected cells with antibodies to the inserted antigen, or any other method available in the art. The construction and testing of the recombinant adenovirus requires no more than ordinary skill in the art.

The recombinant adenoviruses provided by the present invention are useful as therapeutic agents of active immunotherapy for colorectal tumors. The recombinant adenoviruses of the present invention inhibit the growth of a colorectal tumor expressing the specific antigen encoded in the recombinant adenovirus. An effective amount or an immunological response inducing amount of the recombinant adenovirus is the amount that induces humoral and/or cellular responses to the recombinant adenovirus and causes growth arrest or growth inhibition of a colorectal tumor. Such amount varies case-by-case and should be decided by one skilled in the art in light of the specific conditions of a patient.

Usually the amount of the recombinant adenoviruses administered is related to the condition of a patient's immune system, patient's age, the size and duration of the tumor, other medications or therapies applied, and the complications associated with the tumor. A patient's humoral and cellular responses to the recombinant adenovirus can be assessed by any means known in the art, including ELISA, antibody-dependent cytotoxicity (ADCC) assay, and antibody binding inhibition assay. As a guideline, the amount of the

recombinant adenoviruses administered to a patient with colorectal tumor is anywhere from about $10^6$ pfu/day to about $10^{13}$ pfu/day and preferably it is from about $10^7$ pfu/day to about $10^{12}$ pfu/day. The recombinant adenoviruses provided by the present invention may be administered in several injections, preferably one to two rejections.

A recombinant adenovirus encoding different antigens of a colorectal tumor may be administered in an amount similar to that of a recombinant adenovirus encoding a single antigen. A recombinant adenovirus encoding a single colorectal tumor-associated antigen may be administered in combination with another recombinant adenovirus encoding one or more different colorectal tumor-associated antigens.

The recombinant adenoviruses provided by the present invention are also useful as preventive agents for colorectal tumor. The recombinant adenoviruses of the present invention induce protective immunity against colorectal tumors. Such immunity is protective against colorectal tumors carrying the specific antigen encoded by the recombinant adenovirus. However, following a growth reduction or destruction of a tumor carrying an antigen encoded by the recombinant adenovirus, colorectal tumors to be protected against according to the present invention need not express the specific antigen encoded by the recombinant adenovirus. Since most tumor-associated antigens are expressed by less than 100% of the tumor cells within a lesion, recombinant adenoviruses of the present invention are particularly useful to prevent outgrowth of escaping variant tumor cells that do not express the specific antigen encoded in the recombinant adenovirus. An immunity inducing amount of the recombinant adenovirus is the amount that renders protective immunity to a host. Such amount and frequency of administration can be

determined by one skilled in the art via *in vitro* and *in vivo* testing. As a guideline, normally the amount of the recombinant adenovirus for protective immunity or for immunization is anywhere from about $10^6$ pfu/day to about $10^{13}$ pfu/day and preferably it is from about $10^7$ pfu/day to about $10^{12}$ pfu/day. Generally, the immunization is administered in several injections, preferably one to two injections.

The recombinant adenoviruses provided by the present invention are preferably formulated prior to administration. Suitable pharmaceutical formulations are prepared by known procedures using well known and readily available ingredients. In making the compositions suitable for use in the method of the present invention, the active ingredient will usually be mixed with a carrier, or diluted by a carrier, or enclosed within a carrier which may be in the form of a capsule, sachet, paper, or other container. It is preferred that the carrier or a diluent employed keep the recombinant adenoviruses viable and/or stimulate the infection of the viruses. When the carrier serves as a diluent, it may be a semisolid or liquid material which acts as a vehicle, excipient, or medium for the active ingredient. Thus, the compositions can be in the form of sachets, cachets, elixirs, suspensions, solutions, aerosol (in a liquid medium), soft and hard gelatin capsules, suppositories, and sterile injectable solutions for either oral or topical application.

Some examples of carriers, excipient, and diluents include lactose, dextrose, sucrose sorbitol, mannitol, starches, gum acacia, calcium phosphates, alginate, tragacanth, gelatin, calcium silicate, microcrystalline cellulose, polyvinylpyrrolidone, cellulose, methyl cellulose, methyl and propylhydroxybenzoates, talc, and magnesium stearate. Preferably the suitable carriers are physiological saline and phosphate saline.

The formulations can additionally include lubricating agents, wetting agents, and suspending agents, sweetening agents, or flavoring agents. The compositions of the invention may be formulated so as to provide quick, sustained, or delayed release of the recombinant adenoviruses after administration to the patient.

The composition containing one or more of the recombinant adenoviruses of the present invention may be administered orally, intramuscularly, intraperitoneally, intravenously or subcutaneously. The composition may also be applied at the site of the colorectal tumor. The recombinant adenoviruses of the present invention may be administered at a site different from the site of tumor growth and in combination with other therapeutic agents and therapies, *e.g.*, cytokines such as IL-2 and IL-12. A recombinant adenovirus of the present invention may also be administered along with the peptide antigen encoded by the same recombinant adenovirus to enhance the immune response.

The following examples are provided for exemplification purposes only and are not intended to limit the scope of the invention.

**Examples**

**Materials and Methods**

Tumor cells

Murine BALB/c CT26 CRC cells transfected with the human GA733-2 cDNA or control plasmid have been described (22). The transfectants stably express the human GA733-2 antigen and murine MHC class I antigen, both in vitro and in vivo (22). Cells were grown in Eagle's minimal essential medium supplemented with 10% FBS. Human CRC cell line SW1116 was obtained from the American Type Culture Collection (ATCC,

Rockville, MD). Melanoma cell line WM164 and lung carcinoma cell line A549 have been described (23, 24). All cell lines were grown in Leibovitz L-15 medium supplemented with 10% FBS.

Antibodies and Antigens

mAbs CO17-1A, GA733, and M77 bind to different epitopes on the GA733 antigen (1, 25, 26). Anti-CRC mAb CO29-10 binding to an unrelated CRC antigen has been described (27). Recombinant GA733-2E protein derived from baculovirus and used in in vitro lymphocyte stimulation has been described (28).

Recombinant Ad5: Ad5 ΔE3 and Ad5 GA733-2

The early region 3 (E3)-deleted mutant Ad which lacks the XbaI D fragment of Ad (Ad5 ΔE3) has been described (29). The Ad recombinant expressing GA733-2 antigen (Ad5 GA733-2) was constructed using a strategy described by Marshall et al. (30). Briefly, the GA733-2 full-length cDNA (31) under the control of the E3 promoter was inserted into the plasmid pAd5 (m.u. 59-100) in which the E3 coding region was deleted. The recombinant virus was generated by overlap recombination using plasmid pAd5 (m.u. 0-75.9), and the plasmid containing the full-length GA733-2 cDNA (pAd5-GA733-2). The recombinant virus was tested for expression of GA733-2 antigen by immunofluorescence of virus-infected A549 cells using mAb GA733. Both recombinant viruses were grown and titered on A549 cells. Virus purified by CsCl gradient centrifugation (30) was used for immunization of mice.

Immunization and tumor challenge

Six- to 8-week-old female BALB/c mice were used in all immunizations. For

antibody induction, mice were immunized i.p., with 0.75 x $10^8$ pfu of Ad5 GA733-2 or Ad5 ΔE3 once. For induction of proliferative lymphocytes and CTL, mice were immunized i.p. with 5 x $10^7$ pfu of Ad5 GA733-2 or Ad5 ΔE3, three times at 1-month intervals. Ten or 60 days after the last immunization, mice were sacrificed and spleens were removed for CTL or in vitro lymphocyte proliferation assays, respectively. To prevent tumor induction, mice were immunized i.p. with 1.2 x $10^8$ pfu of Ad5 GA733-2 or Ad5 ΔE3 twice at 3-week intervals. Simultaneously with the first immunization or 5 days after the last immunization, mice were challenged with 50 x $10^6$ CT26-GA733-2 tumor cells. To inhibit growth of established tumors, mice were injected with 20 x $10^6$ CT26-GA733-2 tumor cells on day 0 and immunized i.p. with 1.2 x $10^8$ pfu of Ad5 GA733-2 or Ad5 ΔE3 virus on days 5 and 12. Tumor sizes in $mm^2$ (maximal width x length) were measured bi-weekly with a caliper.

Antibody binding assays

Binding of serum antibodies of immunized mice to tumor cells was determined in MHA using SRBC sensitized with mouse anti-SRBC antibody to which goat anti-mouse IgG antibody had been bound (28). Results were expressed as percent tumor cells binding SRBC.

Antibody binding inhibition assays

Inhibition of mAb 17-1A, GA733, M77, or CO29-10 binding to CRC cells by the sera of Ad5 GA733-2- or Ad5 ΔE3-immunized mice was determined in RIA. $^{125}$I-labeled mAb at concentrations showing ~50% maximal binding (10,000 cpm of $^{125}$I-17-1A; 2,500 cpm of $^{125}$I-GA733, M77 or CO29-10 per well of microtiter plate) was added to CRC cells preincubated with various dilutions of sera from immunized mice for 1 h at room

temperature. Inhibition of binding of $^{125}$I-labeled mAb CO17-1A, GA733, M77, and CO29-10 to the cells by the sera was determined relative to buffer controls.

ADCC assays

Sera from immunized mice were analyzed for ADCC reactivity as described (32). In brief, peritoneal exudate cells were isolated from CBA mice injected with 1 ml Brewer's thioglycollate medium 3 days before. Target cells ($1 \times 10^6$) were labeled with 50 mCi [$^3$H]TdR (DuPont NEN, Wilmington, DE) overnight. The next day cells were washed three times, trypsinized, and resuspended at $1 \times 10^4$ cells per well of a microtiter plate. Cells were incubated with mouse sera for 1 h at room temperature, and peritoneal exudate cells were added at various effector-to-target cell ratios. All determinations were done in triplicate. Cell mixtures were incubated for 3 days in a humidified $CO_2$ incubator and [$^3$H]TdR incorporation by the tumor cells was measured in a Matrix 96 ™Direct Beta Counter (Packard, Downers Groves, IL). Results are expressed as percent specific inhibition of [$^3$H]TdR incorporation by target cells (32).

Lymphocyte proliferation assay

Lymphocyte proliferation assays were performed as described (22). Spleens from immunized mice were removed, and cell suspensions were prepared. Cells were stimulated at $1 \times 10^5$/well/0.2 ml of RPMI-1640 medium (Gibco BRL, Grand Island, NY, supplemented with 10% FBS, 50 mg/ml gentamycin, $2 \times 10^{-5}$ M 2-mercaptoethanol, 10 mM HEPES, and 2 mM L-glutamine) with different concentrations of baculovirus-derived GA733-2E antigen (28) for 3 days. Triplicate cultures were then pulsed overnight with 1 mCi per well of [$^3$H]TdR and harvested by a Harvester 96 system (Tomec, Orange, CT).

Radioactivity bound to the cells was determined in a Matrix 96 ™Direct Beta Counter (Packard). Results are expressed as stimulation index (SI).

$$SI = \frac{\text{cpm (experimental cultures)}}{\text{cpm (control cultures)}}$$

Lymphocyte cytotoxicity assay

Spleens from immunized mice were removed, and cell suspensions were made. Splenocytes were stimulated with Ad5 GA733-2- or Ad5 ΔE3-infected autologous spleen cells (responder-to-stimulator ratio = 2) for 5 days in 24-well plates. Lysis of target cells by mouse splenocytes was determined in vitro using a standard $^{51}$Cr-release assay. One x $10^6$ to 5 x $10^6$ target cells were labeled with 100 mCi of $Na^{51}CrO_4$ (DuPont, Boston, MA). Five x $10^3$ cells/well of microtiter plates were plated in 200 ml of RPMI-1640 medium used for T cell stimulation (see above) with various numbers of effector cells (stimulated splenocytes, see above) at effector-to-target ratios of 50, 25, 12.5, 6.2, or 3.1. Wells containing only target cells with either culture medium or 1 M HCl served to determine spontaneous and maximal $^{51}$Cr-release, respectively. Plates were centrifuged at 200 x g for 5 min, incubated at 37°C in a humidified $CO_2$ incubator for 4 h, and centrifuged again. Supernatants (100 ml) were harvested from each well and radioactivity measured in a gamma counter. Percent specific target cell lysis was calculated as:

$$\% \text{ specific lysis} = \frac{\text{cpm (experimental release)} - \text{cpm (spontaneous release)}}{\text{cpm (maximal release)} - \text{cpm (spontaneous release)}} \times 100\%$$

Cytokine determination

Murine IFN-g and IL-4 levels were determined in cell-free supernatants of splenocyte cultures after 3 days of antigen stimulation as described (22). Briefly, cytokine

concentrations were determined by double-determinant RIA using two different mAb binding to different determinants on either of the two cytokines. Recombinant cytokine preparations were used as standards.

Statistical analyses

Experimental and control values were compared using the Student's t-test.

**Example 1**

Humoral immune responses of Ad5 GA733-2 immunized mice

Sera from mice immunized with Ad5 GA733-2 bound specifically to GA733 antigen-positive human and murine CRC cells (Fig. 1), but not to antigen-negative cells (not shown). Sera from mice immunized with the control virus Ad5 ΔE3 did not significantly bind to GA733 antigen-positive cells (Fig. 1). Sera from experimental mice immunized with Ad5 GA733-2 showed significant binding to baculovirus-derived GA733-2E antigen (Fig. 2), but not BSA ($p < 0.05$) (not shown), whereas control sera from mice immunized with Ad5 ΔE3 did not significantly bind to GA733-2E antigen (Fig. 2). Sera from Ad5 GA733-2 immune mice significantly inhibited binding of mAb M77, but not mAb CO17-1A and GA733, to CRC cells SW1116 ($p < 0.05$) (Fig. 3). Antibodies elicited by Ad5 GA733-2 demonstrated low but significant ADCC reactivities against human GA733 antigen-positive CRC cells SW1116 (44% specific lysis; $p < 0.05$ as compared to both pre-immune sera and post-immune sera from mice immunized with the control virus), whereas antigen-negative human melanoma cells were not lysed (not shown).

Ad5 GA733-2 induced antibodies that specifically bound to and lysed

antigen-positive tumor cells in ADCC. Antibody epitope analysis revealed that, of the three epitopes tested (CO17-1A, GA733, and M77), M77 was the only epitope recognized by Ad5 GA733-2 immunized mice. Thus, the humoral immune responses to Ad5 GA733-2 included primarily antibodies directed to CO17-1A and GA733 unrelated epitopes. In contrast, mice immunized with human CRC cells produced antibodies to the CO17-1A and GA733 epitopes (25, 26). Therefore, immunologic presentation of the GA733 antigen expressed by human tumor cells differs from antigen presentation in Ad5 vector.

**Example 2**

Proliferative and cytolytic lymphocyte responses of Ad5 GA733-2 immune mice

Ad5 GA733-2 induced significant and specific lymphocyte proliferative responses following in vitro stimulation of the splenocytes with recombinant GA733-2E protein. Such responses were not observed in Ad5 GA733-2 immune splenocytes stimulated with BSA or in splenocytes from control Ad5 ΔE3 immune mice stimulated with GA733-2E antigen (Fig. 4). Proliferative lymphocyte responses were of the Th1 type as indicated by the significant production of IFN-γ (43.37 ± 12.1 ng/ml; $p < 0.01$), but not IL-4, by splenocytes from Ad5 GA733-2 immune mice following stimulation with baculovirus-derived GA733-2E antigen, as compared to BSA stimulation. IFN-γ production by GA733-2E-stimulated splenocytes derived from Ad5 GA733-2 immunized mice was significantly higher than the IFN-γ production by GA733-2E-stimulated splenocytes of control-immunized mice ($p<0.05$) (results not shown). Proliferating lymphocytes significantly lysed murine CRC cells CT26-GA733-2 as compared to lysis of CT26 control cells. Non-proliferating lymphocytes from mice immunized with control Ad5

ΔE3 induced no significant lysis of CT26-GA733-2 targets (Fig. 5). Ad5 GA733-2 induced antigen-specific cellular immune responses, consisting of proliferating and cytotoxic lymphocytes of the Th1 type.

**Example 3**

Inhibition of tumor growth and induction of protective immunity by Ad5 GA733-2

Mice pre-immunized with Ad5 GA733-2 were protected against subsequent challenge with murine CT26-GA733 cells. Six of 10 mice showed tumor growth 100 days after the start of immunization, whereas all 10 control mice (immunized with Ad5 ΔE3) had large tumors on day 35 and were therefore sacrificed (Fig. 6). When a CT26-GA733-2 tumor challenge was given simultaneously with the Ad5 GA733-2 vaccine, significant growth inhibition was obtained ($p < 0.05$), and only 3 of 10 mice developed tumors by day 50 of the experiment, whereas 8 of 10 control mice developed tumors (Fig. 7*A*). Experimental mice survived significantly longer than control mice ($p < 0.05$) (Fig. 7*B*).

Ad5 GA733-2 significantly enhanced survival of mice with 5-day-old CT26-GA733-2 tumors ($p < 0.05$) (Fig. 8*A*). Five-day-old CT26-GA733-2 tumors are well vascularized, established tumors (results not shown). Survival enhancement was antigen-specific, since growth of antigen-negative CT26 cells was not inhibited by the vaccine (results not shown). Five of the seven mice whose CT26-GA733-2 tumors had completely regressed after Ad5 GA733-2 vaccination (Fig. 8*A*) were challenged on day 98 with GA733 antigen-negative CT26 cells (Fig. 8*B*). Seven naive control mice challenged with the same cells served as control. The previously "cured" mice were protected against challenge with CT26 cells, as demonstrated by significant enhancement of survival of these

mice compared to the control mice ($p < 0.01$) (Fig. 8*B*). None of the five experimental mice had developed CT26 tumors 240 days after challenge, whereas all seven control mice had died of large tumors by day 80. Thus, mice that had rejected the established GA733-2 antigen-positive tumor following immunization with the specific virus were protected from subsequent challenge with the antigen-negative parental tumor. Presumably, the mice that had rejected the transfected tumors due to the development of GA733 antigen-specific immunity also developed immunity to CT26-associated, GA733 antigen-unrelated antigen(s) subsequent to tumor rejection.

Ad5 GA733-2 inhibited the growth of established GA733 antigen-positive, syngeneic CRC cells, resulting in significantly enhanced survival of the mice. Interestingly, mice whose established CT26-GA733-2 tumors had regressed following immunization with Ad5 GA733-2 resisted challenge with GA733-2 antigen-negative parental cells. These results suggest that destruction of CT26-GA733-2 tumor cells by targeting the GA733-2 antigen was followed by induction of immunity to CT26-associated, GA733-2-unrelated antigen, most likely through presentation of tumor debris to the immune system. This secondary vaccine effect or "antigen spreading," which has recently been described by others (33, 34), may explain the powerful anti-tumor effects of the single antigen vaccine observed in our study even against established tumors. Since most tumor-associated antigen, including GA733 (1), are expressed by less than 100% of the cells within a lesion, the induction of immunity to GA733-unrelated CRC antigen demonstrated here might prevent the outgrowth of antigen-negative escape variant tumor cells.

It is believed, without any limitation regarding technical explanation, that the

mechanism(s) underlying tumor growth inhibition according to the present invention is unlikely to be due to antigen-specific CTL alone. For example recombinant vaccinia virus expressing the GA733 antigen protects mice against challenge with CT26-GA733-2 tumors in the absence of detectable antigen-specific CTL induction (our unpublished data). Both recombinant viruses induced antigen-specific, ADCC-reactive antibodies and proliferative lymphocyte responses, either or both of which may have contributed to tumor growth inhibition in vivo. Whereas the lytic antibodies may lyse the tumor cells in conjunction with effector cells such as macrophages (32), the proliferative lymphocytes of Th1 type induced in our study may have activated various effector cells, such as monocytes/macrophages, CTL, and/or eosinophils at the tumor site, leading to tumor destruction.

This study differs in several aspects from previous investigations. First, tumor-associated antigen was used instead of xenogeneic model antigen to demonstrate induction of protective immunity against a human tumor-associated antigen in mice which express an antigen homologue on their normal tissues (20, 21). This is important since most tumor antigens are also expressed on normal tissues. Second, the induction of protective anti-tumor effects by Ad5 GA733-2 did not require cytokines in this study, which represents an advantage in light of the potential toxic side effects of cytokines, especially IL-2. Third, duration of the anti-tumor response, i.e., enhanced survival for >100 days in mice that have been cured of an established tumor is demonstrated. Finally, the vaccine was effective when given at a site different from the site of tumor growth. Thus, the uniqueness of the study is emphasized by the relevance of the animal-model for studies

in cancer patients and the high potency of the vaccine against established tumors. The studies described in the β-galactosidase model system used replication-defective Ad2 with a complete E1A and partial E1B region deletion (16). In the present study, replication-competent, E3 region-deleted Ad5 was used to avoid possible down-modulation of MHC class I by E3 region (17). If desired, a replication-defective adenovirus may used to provide a safer vaccine for humans, and the preliminary studies have shown that this virus is capable of inhibiting tumor growth in mice.

A concern pertaining to the use of recombinant adenovirus as vaccines in patients is whether previous exposure to the virus (almost all individuals are seropositive for Ad5) will negatively affect induction of antigen-specific immunity by recombinant Ad, as shown for vaccinations of humans with recombinant vaccinia viruses (35). However, pre-immunization of mice with control Ad, followed by immunization with Ad expressing β-galactosidase did not inhibit induction of CTL with lytic capability against β-galactosidase-expressing syngeneic tumor targets (18).

A pilot clinical trial in 3 volunteers vaccinated with recombinant Ad hepatitis B virus vaccine administered orally has demonstrated safety and immunogenicity of the Ad (36). The recombinant adenovirus described here are candidate vaccines for CRC patients. It has already been demonstrated that active immunotherapy of CRC patients with Ab2 mimicking the CO17-1A or GA733 epitope induced epitope-specific humoral, cellular, and, possibly, protective immunity. However, single epitope Ab2 vaccines are expected to induce less potent anti-tumor immunity compared to whole antigen vaccines which express multiple, potentially immunogenic epitopes. Ab2 mimicking the GA733 epitope did not

protect mice against challenge with CT26-GA733-2 tumor cells (22), in marked contrast to the potent anti-tumor effects of the Ad5 GA733-2 vaccine described here.

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CLAIMS:

1. A composition useful for the treatment and prevention of colorectal tumors in a human comprising a recombinant adenovirus which contains a DNA fragment operably linked to a viral promoter wherein the DNA fragment encodes an antigen associated with a colorectal tumor and such antigen is expressed when cells are infected by the recombinant adenovirus.

2. The composition of claim 1 wherein the antigen is selected from the group consisting of CO17-1A/GA733 and CEA.

3. The composition of claim 1 wherein the antigen is GA733.

4. The composition of claim 1 wherein the DNA fragment encodes multiple copies of an antigen associated with a colorectal tumor and such antigen is expressed when cells are infected by the recombinant adenovirus.

5. The composition of claim 1 wherein the DNA fragment encodes more than one antigens associated with a colorectal tumor and such antigens are expressed when cells are infected by the recombinant adenovirus.

6. A pharmaceutical composition comprising the recombinant adenovirus of claim 1 in a pharmaceutical carrier.

7. A pharmaceutical composition comprising the recombinant adenovirus of claim 2 in a pharmaceutical carrier.

8. A pharmaceutical composition comprising the recombinant adenovirus of claim 3 in a pharmaceutical carrier.

9. A pharmaceutical composition comprising the recombinant adenovirus of

claim 4 in a pharmaceutical carrier.

10. A pharmaceutical composition comprising the recombinant adenovirus of claim 5 in a pharmaceutical carrier.

11. A method of inhibiting growth of a colorectal tumor comprising administering to a human in need of such treatment an immunological response-inducing amount of the recombinant adenovirus of claim 1, wherein the colorectal tumor expresses the antigen encoded by said recombinant adenovirus .

12. The method of claim 11 comprising administering the recombinant adenovirus of claim 2.

13. The method of claim 11 comprising administering the recombinant adenovirus of claim 3.

14. The method of claim 11 comprising administering the recombinant adenovirus of claim 1 in combination with a cytokine.

15. The method of claim 11 comprising administering the recombinant adenovirus of claim 2 in combination with a cytokine.

16. The method of claim 11 comprising administering the recombinant adenovirus of claim 3 in combination with a cytokine.

17. A method of inducing protective immunity against a colorectal tumor cell in a human comprising administering to a human in need of such treatment an immunity inducing amount of the composition of claim 1.

18. The method of claim 17 wherein the immunity is protective against a colorectal tumor cell which expresses the antigen encoded in the recombinant adenovirus

of the composition.

19. The method of claim 17 comprising administering to a human in need of such treatment an immunity inducing amount of the composition of claim 2.

20. The method of claim 17 comprising administering to a human in need of such treatment an immunity inducing amount of the composition of claim 3.

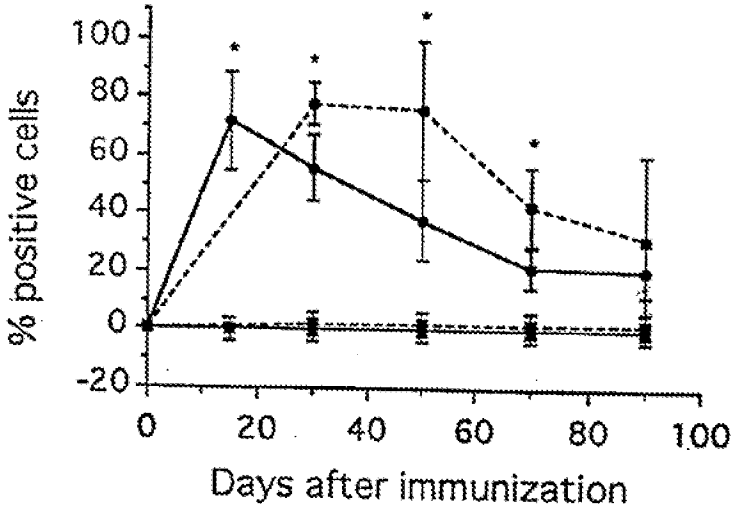

% positive cells
100
80
60
40
20
0
-20
0
20
40
60
80
100
Days after immunization

Fig. 1

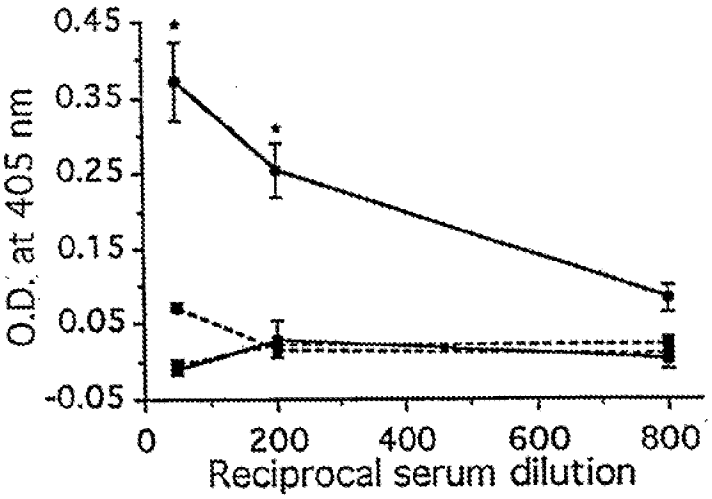

0.45
0.35
0.25
0.15
0.05
-0.05
O.D. at 405 nm
0
200
400
600
800
Reciprocal serum dilution

Fig. 2

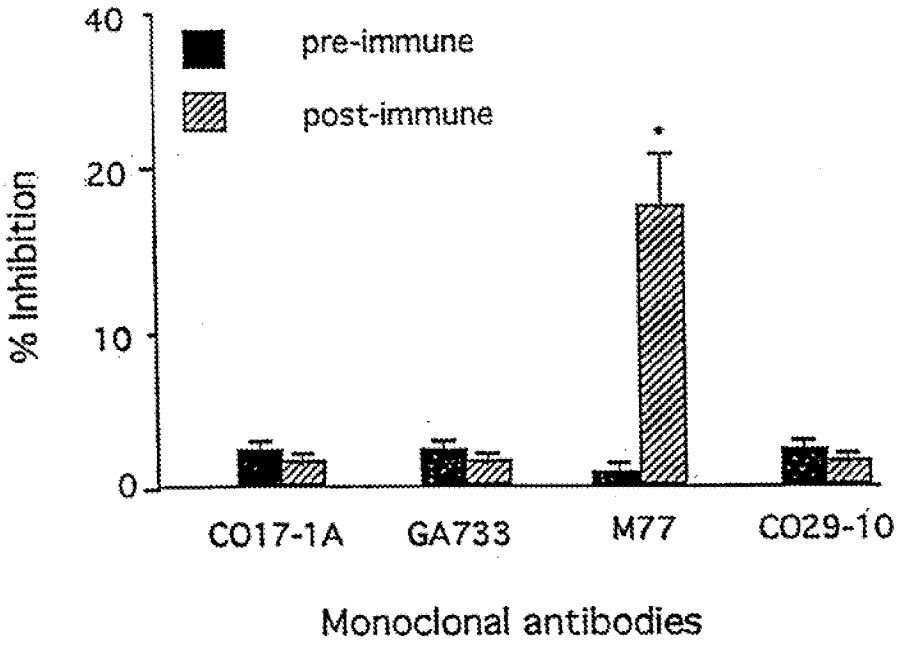
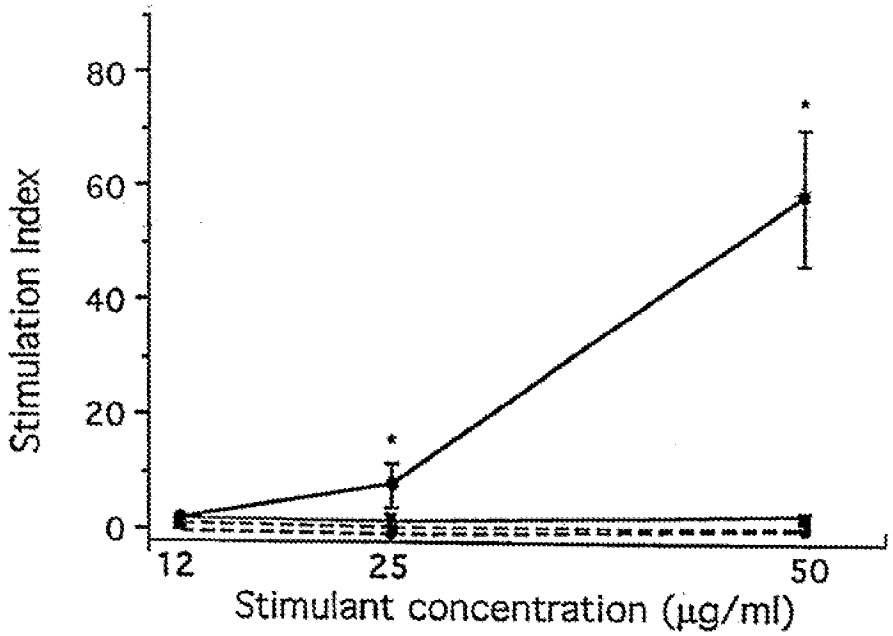

40
20
10
0
% Inhibition
pre-immune
post-immune
*
CO17-1A
GA733
M77
CO29-10
Monoclonal antibodies

Fig. 3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80
60
40
20
0
12
25
50
Stimulant concentration (µg/ml)
*
*

Fig. 4

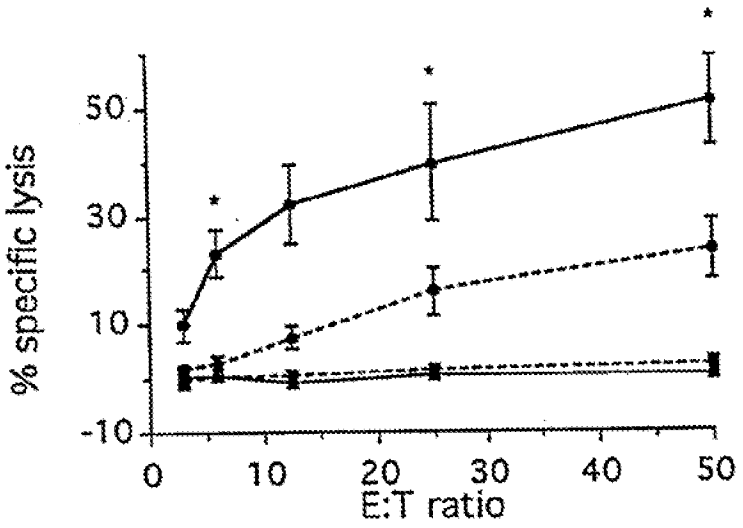



Fig.5

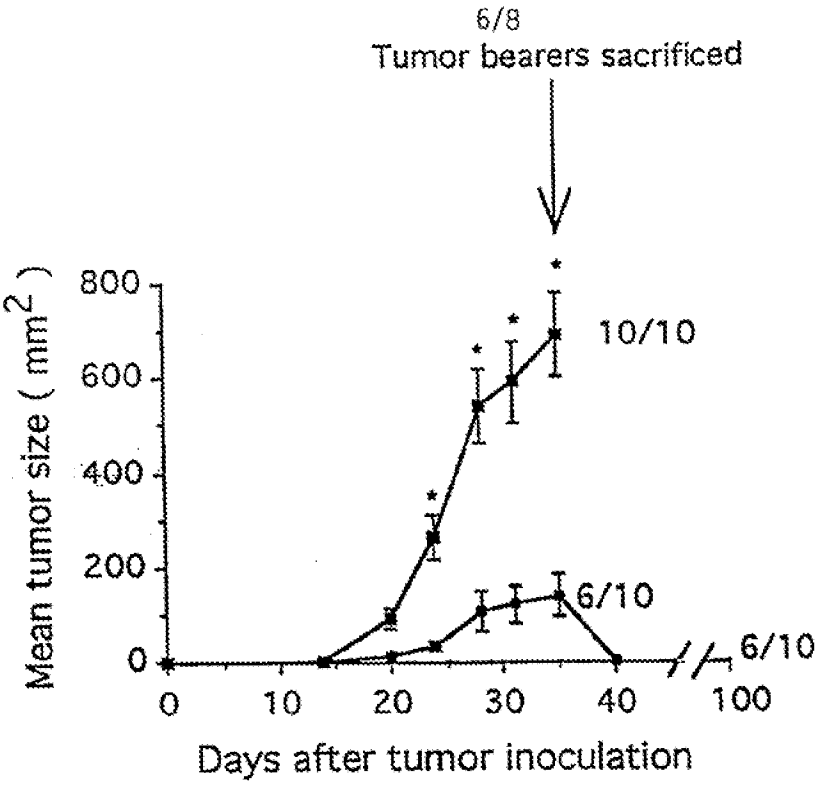
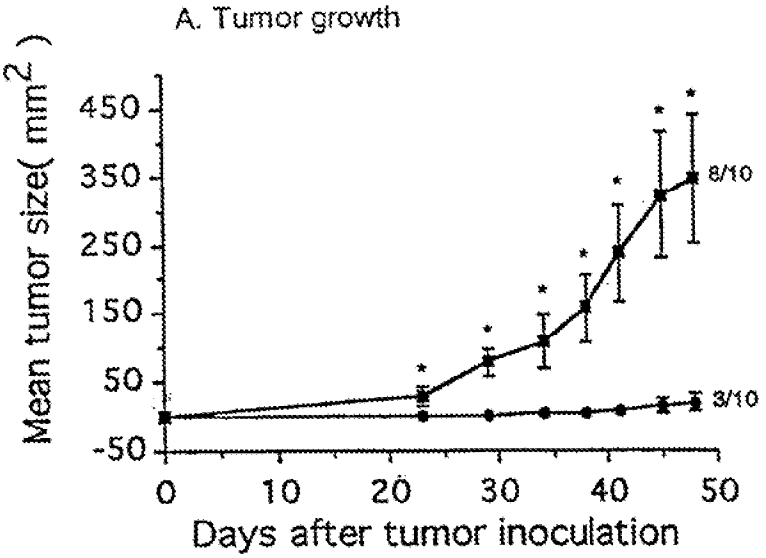

6/8
Tumor bearers sacrificed
Mean tumor size ( mm2 )
800
600
400
200
0
10/10
6/10
6/10
0
10
20
30
40
100
Days after tumor inoculation

Fig.6


A. Tumor growth
Mean tumor size( mm2 )
450
350
250
150
50
-50
0
10
20
30
40
50
Days after tumor inoculation
6/10
3/10

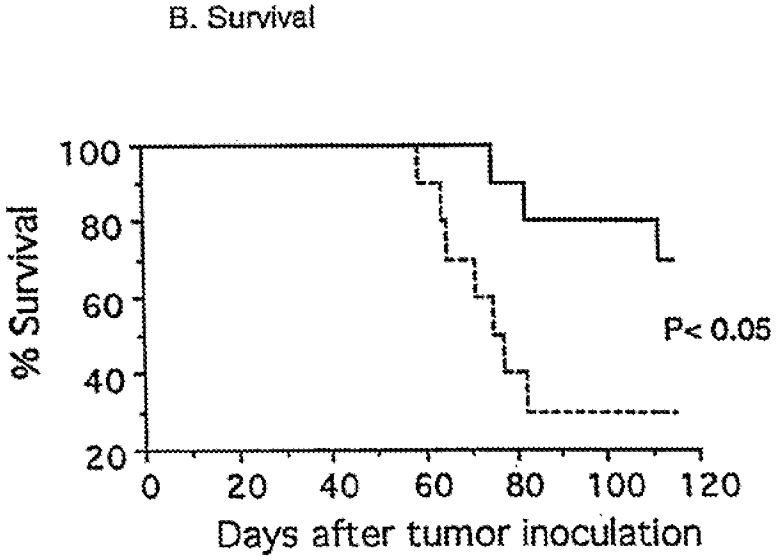

B. Survival
% Survival
100
80
60
40
20
0
20
40
60
80
100
120
Days after tumor inoculation
P< 0.05

Fig.7

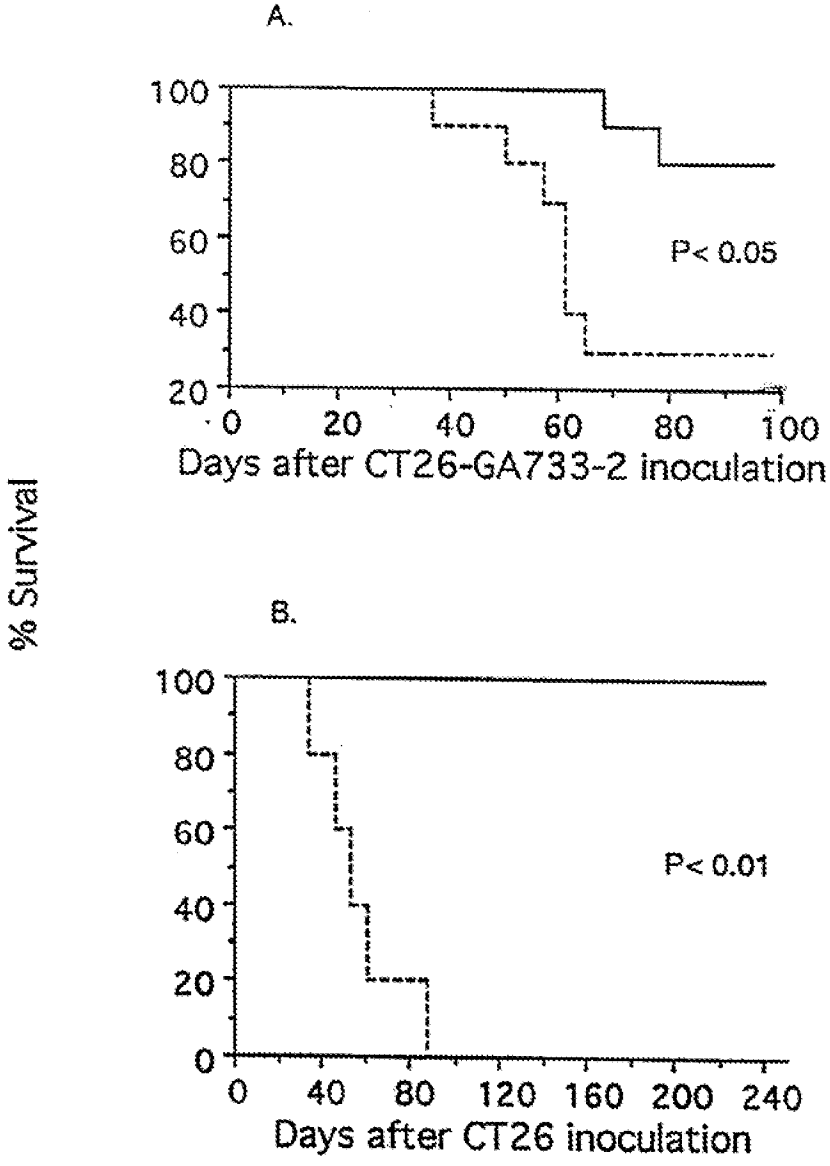

A.
100
80
60
40
20
0
20
40
60
80
100
P< 0.05
Days after CT26-GA733-2 inoculation
% Survival
B.
100
80
60
40
20
0
0
40
80
120
160
200
240
P< 0.01
Days after CT26 inoculation

Fig. 8

# INTERNATIONAL SEARCH REPORT

Inten ..al Application No
PCT/US 97/23783

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/86 A61K48/00 C07K14/82 C12N15/12

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N A61K C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category ° | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| X | LI W. ET AL: "Recombinant human colorectal cancer (CRC) antigen (Ag) CO17-1A-GA733 expressed in adenovirus (AV) inhibits growth of established Ag-positive CRC cells in mice." PROCEEDINGS OF THE AMERICAN ASSOCIATION FOR CANCER RESEARCH ANNUAL MEETING, vol. 37, March 1996, page 479 XP002061092 see abstract #3267 --- | 1-20 |
| X | WO 92 19266 A (US ARMY) 12 November 1992 | 1,2,4-7, 9-12,14, 15,17-19 |
| Y | see the whole document, especially page 9, lines 13-18 --- -/-- | 3,8,13, 16,20 |

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

2

| Date of the actual completion of the international search | Date of mailing of the international search report |
|---|---|
| 1 April 1998 | 23. 04. 1998 |

| Name and mailing address of the ISA | Authorized officer |
|---|---|
| European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016 | Mandl, B |

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# INTERNATIONAL SEARCH REPORT

Inter nal Application No
PCT/US 97/23783

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

| Category ° | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| Y | HERLYN D. ET AL.: "Cloned antigens and antiidiotypes." HYBRIDOMA, vol. 14, no. 2, 1995, pages 159-166, XP002061093 see the whole document | 3,8,13, 16,20 |
| X | WO 96 11279 A (US HEALTH) 18 April 1996 | 1,2,4-7, 9-12,14, 15,17-19 |
| Y | see the whole document, especially page 22, lines 8-11 and page 26, lines 4-19 | 3,8,13, 16,20 |
| Y | SZALA S. ET AL.: "Molecular cloning of cDNA for the carcinoma-associated antigen GA733-2." PROC. NATL. ACAD. SCI. USA, vol. 87, 1990, pages 3542-3546, XP000566331 cited in the application see the whole document | 3,8,13, 16,20 |
| X | RABEN D. ET AL.: "Enhancement of radiolabeled antibody binding and tumor localization through adenoviral transduction of the human carcinoembryonic antigen gene." GENE THERAPY, vol. 3, no. 7, July 1996, pages 567-580, XP002061094 see the whole document | 1,2 |
| A | SEKIGUCHI H ET AL: "Efficient adenovirus-mediated gene transfer into human cancer cell lines derived from digestive tract." INTERNATIONAL JOURNAL OF ONCOLOGY, vol. 8, no. 2, February 1996, pages 283-287, XP002061095 see the whole document | 1-20 |
| P,X | LI W. ET AL.: "Human colorectal cancer (CRC) antigen C017-1A/GA733 encoded by adenovirus inhibits growth of established CRC cells in mice." JOURNAL OF IMMUNOLOGY, vol. 159, no. 2, 15 July 1997, pages 763-769, XP002061096 see the whole document | 1-20 |

2

# INTERNATIONAL SEARCH REPORT

/national application No.
PCT/US 97/23783

**Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)**

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Remark: Although claim(s) 11-20 is(are) directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

**Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)**

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

**Remark on Protest**

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

# INTERNATIONAL SEARCH REPORT

Information on patent family members

Inter nal Application No
PCT/US 97/23783

| Patent document cited in search report | Publication date | Patent family member(s) | Publication date |
|---|---|---|---|
| WO 9219266 A | 12-11-92 | AU 674492 B | 02-01-97 |
| | | AU 2006092 A | 21-12-92 |
| | | CA 2102623 A | 07-11-92 |
| | | EP 0584266 A | 02-03-94 |
| | | JP 6508025 T | 14-09-94 |
| | | US 5698530 A | 16-12-97 |
| WO 9611279 A | 18-04-96 | AU 3998295 A | 02-05-96 |
| | | CA 2201592 A | 18-04-96 |
| | | EP 0789774 A | 20-08-97 |